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Immunology

Chimeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-1A

(recombinant DNA/DNA transfection/cancer immunotherapy)

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ABSTRACT **We have cloned the genomic DNA fragments encoding the heavy and light chain variable regions of monoclonal antibody 17-1A, and we have inserted them into mammalian expression vectors containing genomic DNA segments encoding human $\gamma 3$ and κ constant regions. The transfer of these expression vectors containing mouse–human chimeric immunoglobulin genes into Sp2/0 mouse myeloma cells resulted in the production of functional IgG that retained the specific binding to the surface antigen 17-1A expressed on colorectal carcinoma cells.**

Monoclonal antibodies (mAbs) are highly specific well-characterized reagents. They have found wide applications *in vitro* for immunochemical characterization and quantitation of antigens. Increasingly they are being used *in vivo* for both diagnosis and therapy (1). Their *in vivo* application is limited because in most cases human mAbs of the desired specificity are difficult to prepare (2). Most available mAbs are derived from mouse hybridomas, and their inherent immunogenicity in patients precludes their long-term administration (1). In an attempt to circumvent this problem, chimeric antibodies in which the antigen-specific variable (V) regions of the mouse antibodies are joined to the constant (C) regions of human antibodies have been produced (3, 4). These molecules, which are largely human in composition, should be much less immunogenic and hence should be more suitable for application *in vivo*.

The mouse mAb 17-1A was raised against SW1083 colorectal carcinoma cells and appears to recognize a cancer-associated surface antigen expressed on these cells (5). Here we describe the construction of immunoglobulin genes in which the DNA segments encoding the V regions from the heavy (H) and light (L) chains of this mouse hybridoma were joined to the DNA segments encoding human $\gamma 3$ and κ C regions. Transfection of expression vectors containing these chimeric immunoglobulin genes into mouse myeloma cells resulted in the production of functional chimeric IgG with the same binding specificity as the original hybridoma antibody.

MATERIALS AND METHODS

cDNA Library Construction. Cytoplasmic RNA was extracted from 17-1A cells (the hybridoma cells that produce mAb 17-1A), and poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography. Double-stranded cDNA was synthesized with poly(A)$^+$ RNA as a template, using avian myeloblastosis virus reverse transcriptase and *Escherichia coli* DNA polymerase I. Double-stranded cDNA was treated with S1 nuclease, elongated with deoxycytidine residues, and annealed with oligo(dG)-tailed pUC8 previously cut with *Pst* I (6). The recombinant plasmids were used to transform *E. coli* DH1, and colonies were screened according to the general method described by Maniatis *et al.* (7).

Genomic Library Construction. A genomic DNA library for 17-1A cells was constructed in λ phage vector EMBL3A. High molecular weight DNA was partially digested with restriction endonuclease *Sau*3A and size-fractionated on a 10–40% sucrose density gradient. DNA fragments 18–23 kilobases (kb) long were ligated with λ EMBL3A arms and packaged by using Packagene extracts (Promega Biotec, Madison, WI). The genomic library was screened at a density of 10,000 recombinant plaques per 150-mm-diameter Petri dish. Plaque hybridizations were carried out in 5× SSC at 65°C for 18 hr (1× SSC = 0.15 M NaCl/0.015 M sodium citrate). Final washes were in 0.5× SSC at 65°C. Partial genomic libraries were constructed by using enriched DNA fragments as follows. High molecular weight genomic DNA of 17-1A cells was digested to completion with *Eco*RI and fractionated on a 0.8% agarose gel. DNA fragments of the appropriate size were isolated and ligated with λgt*WES* and λgt11 phage arms. The ligated DNA was packaged and recombinant plaques were screened as described above.

DNA Analysis. Genomic DNA was digested with restriction endonucleases, fractionated by electrophoresis through a 0.7% agarose gel, and blotted onto nitrocellulose (8). Hybridizations were in 5× SSC and 50% (vol/vol) formamide at 37°C for 48 hr. Final washes were in 0.5× SSC at 65°C. Hybridizations using the oligonucleotide as a probe were in 6× SSC at 60°C. The filters were washed in 6× SSC for 1 hr at room temperature and another 30 min at 50°C.

DNA Probes. The mouse $\gamma 2a$ probe is a 4.9-kb *Eco*RI genomic DNA fragment containing the $\gamma 2a$ C region gene. The mouse C_κ probe is a 600-base-pair (bp) *Hin*fI cDNA fragment containing the κ L chain C region sequences. The mouse H chain joining region (J_H) probe is a 2-kb *Bam*HI–*Eco*RI fragment containing both J3 and J4 segments. ^{32}P-labeled probes were prepared by using calf thymus DNA primers (9). Free nucleotides were removed by centrifugation through a Sephadex G-75 minicolumn. The oligodeoxynucleotide 5′-TGTGCAAGAGATGGTCCCTGGTTT-3′ was prepared by using the phosphoramidite method on the Applied Biosystems DNA Synthesizer model 380A. The 24-mer oligodeoxynucleotide probe was prepared by 5′-end labeling with [γ-^{32}P]ATP (7). The reaction mixture was used

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Abbreviations: bp, base pair(s); kb, kilobase(s); mAb, monoclonal antibody; H and L chains, heavy and light immunoglobulin chains; V, C, and J, variable, constant, and joining regions of immunoglobulin chains.

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for hybridizations without prior separation from free nucleotides.

Gene Transfer by Protoplast Fusion. The construction of pSV184neo- and pSV2gpt-derived plasmids carrying the chimeric L and H chain genes, respectively, is described in *Results*. *E. coli* HB101 harboring both plasmids were grown in the presence of ampicillin and chloramphenicol. The plasmid copy number was amplified with spectinomycin at 100 μg/ml. Protoplasts were prepared and fused to mouse myeloma Sp2/0 cells according to Oi *et al.* (10). Transformants were selected in Dulbecco's modified Eagle's medium containing antibiotic G418 at 0.8 mg/ml, supplemented with 15% fetal calf serum. The SG3/5 cell line was maintained in the above medium plus xanthine at 50 μg/ml, hypoxanthine at 4 μg/ml, and mycophenolic acid at 0.8 μg/ml. The frequency of G418-resistant transformants was approximately 5×10^{-6}.

Analysis of Immunoglobulin by Biosynthetic Labeling and Immunoprecipitation. Cells were labeled for 3 hr in methionine-free RPMI 1640 medium to which [^{35}S]methionine had been added at 25 μCi/ml (1 Ci = 37 GBq). Affinity-purified goat antibody to human κ L chain (Southern Biotechnology Associates, Birmingham, AL) and goat antibody to the Fc fragment of human IgG (Jackson ImmunoResearch, Avondale, PA) were used for immunoprecipitations (11). Both cytoplasmic and secreted antibodies were analyzed on a $NaDodSO_4$/5% polyacrylamide gel in phosphate buffer under nonreducing conditions (12). The gel was treated with an autoradiography enhancer, EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 film.

Quantitation of Antibody Production. Tissue culture supernatant was analyzed for IgG protein content by particle concentration fluorescence immunoassay (13) using standard curves generated with purified IgG. Concentration of mAb 17-1A was determined by using polystyrene beads coated with goat antibody to mouse Fab and fluorescein-conjugated goat antibody to mouse Fab. Concentration of chimeric antibody SG3/5 was determined by using polystyrene beads coated with goat antibody to the Fc fragment of human IgG and fluorescein-conjugated goat antibody to human IgG Fc fragment. The assays were carried out with an automated instrument (Pandex Laboratories, Mundelein, IL).

Radioiodination of mAb 17-1A. mAb 17-1A was purified from ascites fluid by chromatography on staphylococcal protein A-Sepharose. Bound IgG was eluted with 0.05 M sodium citrate buffer at pH 3.5. Purified mAb 17-1A was labeled with 1.4 mM chloramine-T, using 1 mCi of Na^{125}I. After 3 min, the reaction was quenched with excess ascorbic acid. Free iodine was removed with a prepacked PD-10 column (Pharmacia). Specific activity was typically 10,000 cpm/ng of protein.

Binding Inhibition Assay. Tissue culture supernatant of 17-1A or SG3/5 cells was concentrated with a Diaflo YM100 ultrafiltration membrane (Amicon). Monolayer SW1116 colorectal carcinoma cells were treated with trypsin and washed in Dulbecco's phosphate-buffered saline (PBS). Then 5×10^5 cells were incubated with 10^5 cpm of radioiodinated mAb 17-1A and culture supernatant containing the competing antibody in a final volume of 100 μl. Incubation was at room temperature for 2 hr in a shaker at 140 rpm. The cells were washed with PBS and cell-bound radioactivity was measured in a γ counter.

RESULTS

Isolation and Sequencing of the L and H Chain cDNA of mAb 17-1A. A cDNA library was prepared for the 17-1A cells by using plasmid vector pUC8. Recombinant colonies were screened with the mouse C_κ probe and the mouse $C_{\gamma 2a}$ probe to isolate the L and H chain clones, respectively. A total of 7500 colonies were screened, of which approximately 250 contained C_κ sequences and 150 contained $C_{\gamma 2a}$ sequences. Plasmid DNA prepared from some of these positive colonies was digested with *Pst* I to compare the size of the cDNA inserts. The L chain clone, pMκ-9, and the H chain clone, pMγ2a-1, were chosen for nucleotide sequencing. The nucleotide sequences and the predicted amino acid sequences for the 5′ region containing the leader peptide and the V region are shown in Fig. 1. Comparison of the nucleotide sequences near the 3′ end of the V_L gene with those of the J_κ locus (14) showed that nucleotides 427–462 in Fig. 1*A* were identical to the sequences encoding the $J_{\kappa 2}$ segment. The functional 17-1A L chain gene thus resulted from a V–J joining event that juxtaposed the V gene to the $J_{\kappa 2}$ exon during a rearrangement within the L chain locus. Similarly, we concluded that the 17-1A H chain gene used the J_{H3} segment, since nucleotides 370–414 of Fig. 1*B* were identical to the sequences encoding the J_{H3} segment (15).

A V_L probe was derived from pMκ-9 as a *Bam*HI–*Pvu* I fragment that contained the first 320 nucleotides of the L chain gene (Fig. 1*A*). The V_H probes derived from pMγ2a-1 were two *Pst* I fragments: the 228-bp V_H1 and the 132-bp V_H2 probes corresponding to nucleotide 52–280 and 281–412, respectively (Fig. 1*B*). These V region probes were used in subsequent experiments to characterize the genomic DNA fragments containing functionally rearranged genes.

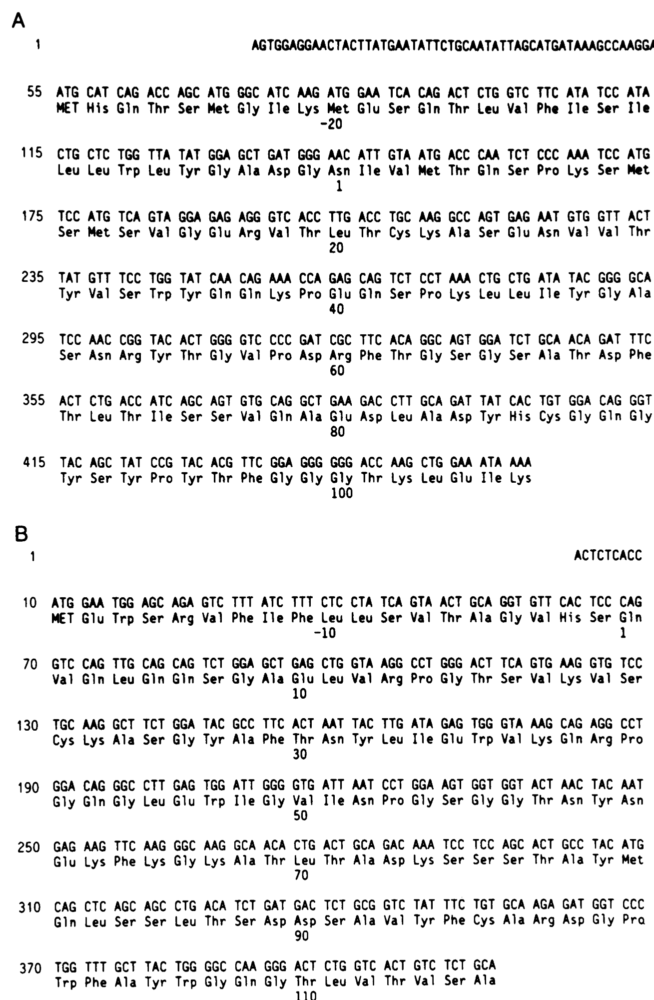

FIG. 1. Nucleotide sequences of the mAb 17-1A functional L chain (*A*) and H chain (*B*) genes and the predicted amino acid sequences. The C regions are not shown. Amino acid residues are numbered, and negative numbers refer to the amino acids in the leader peptide.

Cloning of the Genomic DNA Fragment Containing the Functionally Rearranged L and H Chain Genes of 17-1A. A genomic DNA library was prepared for the 17-1A cells. Approximately 200,000 λ phage recombinants were screened with the mouse C_κ probe. Three positive clones were obtained. One of these clones, λκ4, was shown to contain the V region sequences by restriction mapping and Southern analysis using the V_L probe derived from the cDNA clone, pMκ-9. A 4.2-kb *Hin*dIII genomic DNA fragment containing 1.5 kb of the 5′ flanking region and the sequences encoding the leader peptide and the V gene was subcloned in pUC18 and designated $pV_\kappa 4.2H$. This subclone was used in the subsequent construction of the mouse–human chimeric L chain gene.

When the 17-1A genomic library was screened with the mouse Cγ2a probe, two positive clones were obtained, but neither of these contained the V_H gene of the cDNA clone, pMγ2a-1. An alternative approach was taken to clone the V_H gene. During the gene rearrangement that is required for immunoglobulin gene expression, the V gene is always moved next to a J segment. As illustrated in Fig. 2*B*, the J_H probe can therefore be used to detect rearranged V_H genes when the appropriate restriction enzyme is used. Fig. 2*A* shows the Southern analysis of rearranged fragments containing J_H sequences. DNA of 17-1A cells showed two rearranged *Eco*RI fragments at 7.4 and 3.8 kb in addition to the band characteristic of the fusion partner, P3. The P3 cells had two rearranged V_H genes that comigrated at 6 kb. One of the two bands of 17-1A represented the functional rearrangement, while the other was the product of an aberrant gene rearrangement in the H chain locus. Using the mouse J_H probe, both genes were cloned from phage genomic libraries constructed with enriched DNA fragments of appropriate sizes. Clone $\lambda V_H 7.4E$ was isolated from a λgt*WES* library and the *Eco*RI insert was found to comigrate with the 7.4-kb rearranged fragment, as indicated with an arrowhead in Fig. 2*A*. Clone $\lambda V_H 3.8E$ was isolated from a λgt11 library and its *Eco*RI insert comigrated with the 3.8-kb band, as indicated with a solid circle in Fig. 2*A*.

To identify the genomic counterpart of the functional H chain gene, both $\lambda V_H 7.4E$ and $\lambda V_H 3.8E$ were digested with *Pst* I and hybridized with the mixed V_H1 and V_H2 probes derived from the cDNA clone pMγ2a-1. $\lambda V_H 7.4E$ contained two *Pst* I fragments, 300 and 130 bp. Since the V_H1 probe contained sequences encoding the amino acid residue −5, which is just upstream of the intron/exon boundary within the leader peptide, the difference between the longer *Pst* I fragment in the genomic clone (300 bp) and that in the cDNA clone (228 bp) suggested the size of the intron between the leader peptide and the variable region gene to be 70 bp. Clone $\lambda V_H 3.8E$ contained different length *Pst* I fragments and did not cross-hybridize with V_H1 and V_H2 probes. To further verify that $\lambda V_H 7.4E$ is the genomic counterpart of the cDNA clone pMγ2a-1, a 24-mer oligonucleotide probe corresponding to nucleotides 352–375 (Fig. 1*B*) was synthesized; it was found to specifically hybridize to the 130 bp *Pst* I fragment of $\lambda V_H 7.4E$ (data not shown). This oligomer contained sequences encoding the CDR3 region and should be characteristic of this V gene. $\lambda V_H 7.4E$ was used in the construction of the mouse–human chimeric H chain gene.

Vectors and Expression System. The functionally rearranged L and H chain V genes isolated from the 17-1A cells were joined to human κ and γ3 C region genes in expression vectors containing dominant selectable markers, *neo* (16) and *gpt* (17), respectively. To construct the desired chimeric gene, the *Hin*dIII fragment of pSV184ΔHneoDNSV$_L$-hC$_\kappa$ (18) containing the dansyl-specific V_L gene was replaced with the 4.2-kb *Hin*dIII fragment containing the L chain gene of 17-1A derived from the clone $pV_\kappa 4.2H$. The structure of pSV184ΔHneo17-1AV$_\kappa$hC$_\kappa$ is shown in Fig. 3*A*. The H chain vector was constructed by replacing the *Eco*RI fragment in the pSV2ΔHgptDNSV$_H$-hC$_{\gamma3}$ plasmid containing the dansyl-specific V_H gene with the 7.4-kb *Eco*RI fragment containing the functionally rearranged 17-1A H chain gene derived from the genomic clone $\lambda V_H 7.4E$. The resulting plasmid is designated pSV2ΔHgpt17-1AV$_H$-hC$_{\gamma3}$ (Fig. 3*B*).

The L and H chain vectors shown in Fig. 3 were used to transfect mouse myeloma cells. The pACYC184 plasmid confers chloramphenicol resistance and the pBR plasmid confers ampicillin resistance; it is therefore possible to transform *E. coli* with both L and H chain plasmids and select cells expressing dual drug resistance phenotypes. To trans-

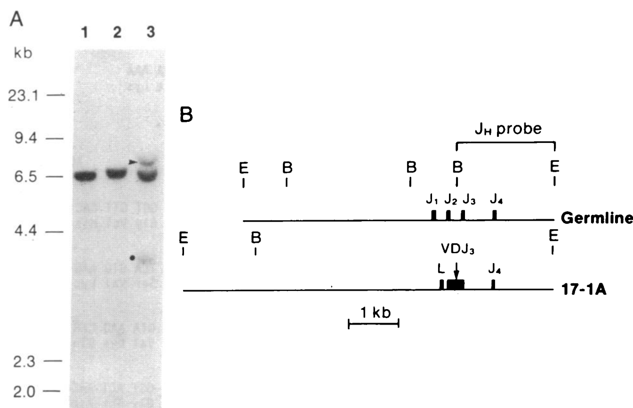

FIG. 2. (*A*) Southern blot analysis. Ten micrograms of genomic DNA was digested with *Eco*RI, fractionated on 0.7% agarose, and transferred to nitrocellulose, and the bound DNA was hybridized with the mouse J_H probe. Lane 1, mouse liver DNA; lane 2, P3, a mouse plasmacytoma cell line; lane 3, 17-1A, a hybridoma cell line derived by using P3 as a fusion partner. The arrowhead indicates the rearranged fragment containing the functional V_H gene of 17-1A. The solid circle indicates an additional rearrangement in the H chain locus. (*B*) Restriction maps of the germline J_H region and the functionally rearranged 17-1A V_H gene. Exons are represented with solid boxes. The bracket above the germline restriction map indicates the J_H probe used. Only *Eco*RI (E) and *Bam*HI (B) restriction sites are shown.

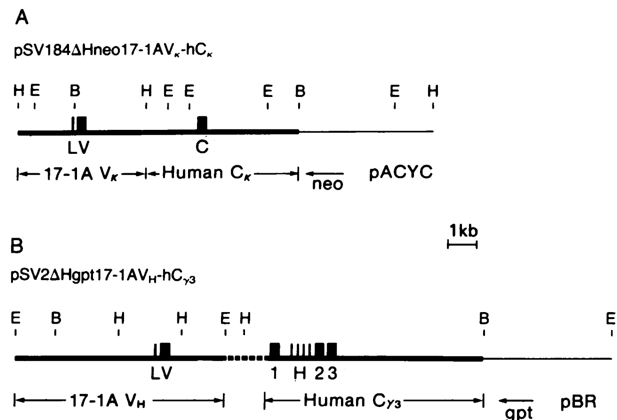

FIG. 3. Structure of the chimeric L and H chain vectors. (*A*) pSV184ΔHneo17-1AV$_\kappa$-hC$_\kappa$; (*B*) pSV2ΔHgpt17-1AV$_H$-hC$_{\gamma3}$. Immunoglobulin DNA is represented by thick lines and exons by solid boxes. Vector sequences are shown as thin lines. The transcriptional directions of the *neo* and *gpt* genes are as indicated. The ranges of the DNA sequences are indicated with two-way horizontal arrows. L, leader exon; V, VJ or VDJ exon; C, constant region exon; H, hinge exon; 1, 2, and 3, exons encoding other domains of the H chain gene. Broken line between 17-1AV$_H$ and human C$_{\gamma3}$ gene domains represents residual S107 intervening sequences (8) carried over during the derivation of this plasmid. Restriction endonuclease abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

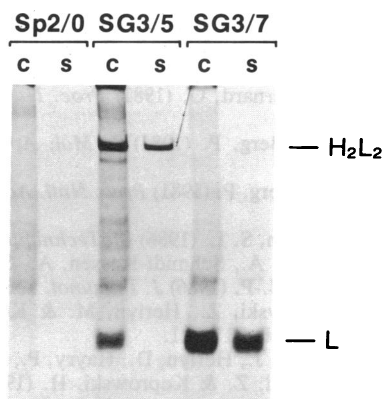

FIG. 4. Analysis of chimeric immunoglobulin proteins in the transfected Sp2/0 cell lines SG3/5 and SG3/7. Cells were labeled for 3 hr with [^{35}S]methionine. Immunoglobulins from cell extracts and culture supernatants were precipitated with antibody to human κ chain and electrophoresed on a $NaDodSO_4$/5% polyacrylamide gel containing phosphate buffer under nonreducing conditions. Lanes c, cytoplasmic; lanes s, secreted. The positions of the tetrameric H_2L_2 protein and the L chain protein are indicated.

fect the chimeric genes into mouse myeloma cells, *E. coli* harboring both plasmids were converted to protoplasts and fused with a nonproducing mouse myeloma cell line, Sp2/0 (3, 10). After protoplast fusion, the transfected cells were initially selected only for *neo* gene activity in the presence of G418. The stable transformant lines were subsequently carried in medium containing both G418 and mycophenolic acid.

Analysis of Chimeric Antibody Production. Seven stable transformants were established and analyzed. Two of these clones produced both H and L chain proteins, two produced only L chain proteins, and three did not produce any detectable immunoglobulin. Fig. 4 shows the result of a biosynthetic labeling experiment for the SG3/5 cell line, which produced both H and L chain protein. Intermediates in immunoglobulin assembly were detected in the cell extract; however, only fully assembled H_2L_2 molecules were secreted into the culture medium. Similar results were obtained by using the Sepharose-bound anti-human IgG Fc antibody in the immunoprecipitations (data not shown). The IgG concentration in the culture supernatant of the SG3/5 cell line was 20 μg/ml as measured by particle concentration fluorescence immunoassay (13). The SG3/7 cells, which also showed resistance to both G418 and mycophenolic acid, produced and secreted only the L chain proteins (Fig. 4), indicating that the integration of the plasmids pSV2ΔHgpt17-1AV$_H$-hC$_{\gamma3}$ and pSV184ΔHneo17-1AV$_\kappa$-hC$_\kappa$ yielded functional *gpt*, *neo*, and chimeric L chain genes. However, the H chain protein was not produced, probably due to changes within the immunoglobulin gene or its control regions.

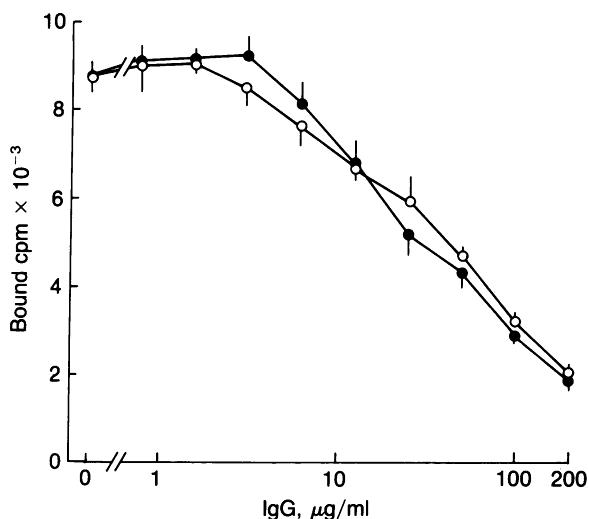

FIG. 5. Binding of radioiodinated mAb 17-1A to SW1116 cells in the presence of culture supernatants from 17-1A (●) or SG3/5 (○) cells. Each point was determined in triplicate; bars indicate SD.

Binding Specificity of Chimeric Antibody. Binding inhibition assays were used to demonstrate that the chimeric mAb SG3/5 bound to the same surface antigen of the SW1116 colorectal carcinoma cells as mAb 17-1A. As shown in Fig. 5, curves for the binding of radioiodinated mAb 17-1A to SW1116 cells in competition with 17-1A itself and SG3/5 were superimposable. Thus, the replacement of mouse C regions in mAb 17-1A with human C regions did not affect its antigen-binding affinity or specificity.

DISCUSSION

mAb 17-1A has recently been demonstrated to recognize a 41-kDa glycoprotein surface antigen found on human colorectal carcinoma cells (19). In nude mice grafted with human colorectal carcinoma cells, mAb 17-1A was shown to have anti-tumor activity (20). mAb 17-1A has been used in immunotherapy trials to treat patients with gastrointestinal cancer (21, 22). In some cases, treatment with mAb 17-1A resulted in a partial or complete regression of tumor masses (23, **). Although mAb 17-1A had no toxic effect at doses up to 400 mg per infusion, repeated administration of the mouse antibody often induced human anti-mouse immunoglobulin antibodies, rendering the mouse antibodies ineffective for further therapy (24). Since human antibodies of the appropriate specificity are not available, chimeric antibodies with V regions identical to those of the mouse hybridoma and human C regions may provide antibodies of the appropriate specificity that are less immunogenic than the completely mouse antibodies.

To test this possibility, we have joined the DNA segments encoding the mouse V regions from the antibody specific for the cancer-associated 17-1A surface antigen to the DNA segments encoding human $\gamma3$ and κ C regions. These DNA segments, when introduced into a nonproducing variant of a mouse myeloma cell line, directed the synthesis of a complete immunoglobulin molecule, which was secreted by the mouse myeloma cell. The chimeric antibody molecules exhibited binding characteristics identical to those of the starting mouse molecules. The level of synthesis was sufficient to permit isolation of large quantities of materials from culture supernatants.

Initial studies had shown that chimeric antibodies specific for hapten retained their ability to react with the antigen (25–28). In the current studies, we extend these observations by demonstrating that a mouse–human chimeric antibody retains its ability to react with a carcinoma-associated antigen. These chimeric antibodies should be much less immunogenic than the mouse antibodies. In addition, the human C regions of the chimeric antibodies may more effectively carry out the human effector functions. With chimeric antibodies, one is not limited to using antibodies of only a single isotype, and it is possible to produce chimeric antibodies with the 17-1A-derived V regions linked to the human $\gamma1$, $\gamma2$, and $\gamma4$ C regions. Such antibodies will be important in studying the role of different IgG subclasses in mediating certain immune functions. Furthermore, the ability to genetically engineer

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changes in the DNA segments enables one to produce antibody molecules with "tailor-made" effector functions (4, 29). The most effective antibodies can then be produced for use in immunotherapy. The 17-1A system is ideal for these studies since there is a broad background of information on the therapeutic efficacy of the mouse antibodies (**).

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